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(54) **Title:** PEPTIDE ANTAGONISTS DERIVED FROM THE TRANSMEMBRANE DOMAINS OF G PROTEIN-COUPLED RECEPTORS

(57) Abstract

This invention relates to peptides and peptidomimetic compounds that act as antagonists against G protein-coupled receptors (GPCRs). Novel short peptides, derived from the transmembrane domains of GPCRs, ranging in size from about 15-20 amino acid residues, can be used as model peptides (peptide-leads) to design novel peptides and peptidiomimetic compounds that antagonize the activity of the same or closely related GPCRs from which they are derived. A lead peptide which may also be a preferred peptide antagonist for the human β2-adrenergic receptor is NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH.

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PEPTIDE ANTAGONISTS DERIVED FROM THE TRANSMEMBRANE DOMAINS OF G PROTEIN-COUPLED RECEPTORS

FIELD OF THE INVENTION

The present invention relates to novel peptides and/or peptidomimetic compounds that can be used as antagonists of GPCRs.

BACKGROUND OF THE INVENTION

The class of receptors known as G protein-linked receptors (GPCRs) are typically characterized by a 7-helix organization, whereby the receptor protein is believed to traverse the membrane seven times. GPCRs share a common signalling mechanism, whereby signal transduction across the membrane involves intracellular transducer elements known as G proteins. When a chemical messenger binds to a specific site on the extracellular surface of the receptor, the conformation of the receptor changes so that it can interact with and activate a G protein. This causes a molecule, guanosine diphosphate (GDP), that is bound to the surface of the G protein, to be replaced by another molecule, guanosine triphosphate (GTP), triggering another conformational change in the G protein. When GTP is bound to its surface, the G protein regulates the activity of an effector. These effectors include enzymes such as adenylyl cyclase and phospholipase C, channels that are specific for calcium ions (Ca^{2+}), potassium ions (K^+), or sodium ions (Na^+) and certain transport proteins.

In general, activation of GPCRs by transmitters will induce one or another of the following effector responses:. activation of adenylyl cyclase, inhibition of adenylyl cyclase or

stimulation of phospholipase C activity. When the effector adenylyl cyclase is either activated or inhibited it produces changes in the concentration of the molecule cyclic adenosine monophosphate (cAMP). Another effector, phospholipase C, causes one molecule of phosphatidylinositol-bisphosphate (PIP_2) to be cleaved into one molecule each of inositol triphosphate (IP_3) and diacylglycerol (DAG); IP_3 then causes calcium ions (Ca^{2+}) to be released into the cytoplasm. Alterations in cellular levels of cAMP and Ca^{2+} are two of the most important intracellular messages that in turn act to alter the behaviour of other target proteins in the cell.

Receptors may be classified according to the type of signaling pathway they activate in cells. This occurs at the level of the G proteins, which detect and direct signals from diverse receptors to the appropriate effector-response pathway. The three main groups of G proteins are: Gs-like, which mediate adenylyl cyclase activation; Gi-like, which mediate inhibition of adenylyl cyclase; and Gq-like, which mediate activation of phosphoplipase C. Since one receptor can activate many G proteins, the signal can be greatly amplified through this signal transduction pathway.

A wide variety of chemical messengers involved in regulating key functions in the body act through GPCRs. These include neurotransmitters such as dopamine, acetylcholine and serotonin, hormones of the endocrine system such as somatostatin, glucagon and adrenocorticotropin. and lipid mediators such as prostaglandins and leukotrienes. Over one hundred different GPCRs have been identified in humans, and many more are expected to be discovered. All of these receptors are believed to utilize one of the three principal G protein-effector signaling pathways (stimulation or inhibition of adenylyl cyclase or

activation of phospholipase C).

Examples of G Protein-Coupled Neurotransmitter Receptors

Believed to Regulate Adenylyl Cycase

Inhibits	Stimulates	Neurotransmitter
m_2, m_4, m_5		Acetylcholine
A_1, A_2		Adenosine
CRF-R		Corticotropin-releasing factor
CbR		Cannabinoids
D_2	D_1, D_5	Dopamine
	H_2	Histamine
Y_1, Y_2, Y_3		Neuropeptide Y
α_2-AR	β_1-AR, β_2-AR	Norepinephrine, epinephrine
μ, δ, κ	κ	Opioids
5-HT_{1A}, 5-HT_{1B}	5-HT_4	Serotonin
5-HT_{1D}		

Adrenergic receptors have been extensively studied. In fact the human β_2 receptor has been used as a model to illustrate the common structural features shared by members of the G protein-coupled receptor family (Kobilka, G., *Annu Rev. Neurosci.*, 15:87-114, 1992). Evidence from biochemical (Dohlman et al., *J. Biol. Chem.*, 262:14282-14288, 1987) and immunologic studies of the topology of the β_2 adrenergic receptor (Wang et al. *J. Biol.*

Chem. 264: 14424-14431. 1989) supports the model that most GPCRs comprise seven membrane spanning domains (However. it must be noted that there are some G protein activating receptors that are not members of the seven membrane spanning family. For example. the receptor for insulin-like growth factor II directly activates G_{i-2}, has only a single membrane spanning domain).

The ability to regulate the in vivo activity of GPCR has many significant clinical implications. More importantly, the ability to selectively inhibit GPCR functions is highly desirable so that precise drug therapy can be effected minimizing or eliminating potential side effects arising from cross-activity with other receptors.

Compounds that selectively antagonise the activities of GPCRs would have great utility for many industries whose goal is to develop chemical substances that interact with G protein-coupled receptors. Since G protein-coupled receptors are ubiquitous and widely used in nature to transmit cellular signals, this invention has utility for different industries including: the pharmaceutical industry, the pest-control industry, the aquaculture industry, the food industry and the fragrance industry.

The pharmaceutical sector is particularly interested in the potential therapeutic applications of GPCR antagonists for pathologies associated with depression and psychosis. Moreover. there are several genetic diseases shown to be associated with mutations in GPCRs. Drugs which possess receptor downregulating activity could have particular therapeutic relevance for such conditions. In fact, many existing therapeutic drugs act by directly modifying the

function of GPCR, although in most cases. these drugs exert their effects on receptor function by binding to the same site on the receptor component as the natural chemical messenger.

The background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceeding information constitutes prior art against the present invention. Moreover, publications referred to in the following discussion are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

The present invention resides in the discovery that novel compounds modelled on the transmembrane region of GPCRs are potent GPCR antagonists. Specifically, the present invention relates to novel peptides and/or peptidomimetic compounds that are modeled on the transmembrane region of GPCRs and that function as antagonists against the same receptor from which they were derived. A working example is provided. based on residues 276 - 296 of the β_2-adrenergic receptor, wherein the peptide inhibits agonist promoted stimulation of adenylyl cyclase activity.

The peptide and peptidiomimetic antagonists of this invention may be utilized in compositions and methods for specifically controlling certain GPCR functions. The invention also involves a process for antagonizing GPCR activity in mammals which

comprises: administering to a subject an effective amount of the novel compoujd to antagonise GPCR activity.

A further embodiment involves a pharmaceutical preparation for treating disease and psychoses which comprises administering a pharmaceutically effective amount of the novel peptide or peptidiomimetic compound sufficient to antagonize GPCR with a suitable pharmaceutical carrier.

Another aspect of this invention involves generating peptides and peptidiomimetic compounds that are useful for in vito and in vivo studies of GPCRs.

Due to the fact that the antagonists molecules of the present invention may be prepared by chemical synthesis techniques, commercially feasible amounts may be produced inexpensively. Moreover, because the antagonist molecules of the present invention are relatively small and may be peptidergic in nature, they are less likely to stimulate an undesirable immune response in patients treated with them.

BRIEF DESCRIPTION OF THE DRAWINGS

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following preferred embodiments of the invention taken in connection with the accompanying drawings in which:

Figure 1 demonstrates effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in Sf9 cells. **A.** Membrane preparations derived from β2AR expressing Sf9 cells were either not treated (open circles), or treated with TM VI peptide (closed squares). control peptide TM VI Ala (closed circles), or second control peptide from TM VII of the D2 dopamine receptor (open triangles). Isoproterenol stimulated adenylyl cyclase activity was then assessed for these membranes. Data are expressed relative to the maximal stimulation obtained with the untreated membranes and represent mean +/- SEM for 8 independent experiments. Peptides were used at a concentration of 0.15 $\mu g/\mu l$. **B.** Effects of TM VI peptide (hatched bars) or vehicle alone (open bars) on basal (n = 13), maximal isoproterenol-stimulated (ISO, n = 13), forskolin-mediated (FSK, n = 13) and NaF-stimulated (n = 6) adenylyl cyclase activity was investigated. Data are expressed as pmol cAMP produced per mg membrane protein per minute +/- SEM. Statistical significance of the difference are indicated by an asterisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. None of the control peptides discussed in figure 2 had effects on adenylyl cyclase stimulation in β_2AR expressing cells nor did any of the peptides have effects on adenylyl cyclase stimulation in Sf9 cells which were infected with the wildtype baculovirus (data not shown). **C,** Effects of increasing concentrations of peptide on isoproterenol and dopamine stimulated adenylyl cyclase activity were also investigated. Membranes were prepared from Sf9 cells expressing either the human β_2AR (open circles) or the human D1 dopamine receptor (closed circles). Adenylyl cyclase activity was measured using maximally stimulating concentrations of either isoproterenol (10^{-4} M) or dopamine (10^{-4} M) in the presence of peptide concentrations ranging from 10-8 to 10-4 M. Data were analyzed by non-linear least squares regression using SigmaPlot (Jandel

Scientific). The data are expressed as the mean +/- SEM (n = 3).

Figure 2 depicts effects of TM VI peptide on β_2AR expressed in mammalian cells. **A.** Effect of 0.15 ug/ul TM VI peptide (hatched bars) or vehicle (open bars) on basal (n=2), maximal isoproterenol-stimulated (ISO, n=2) forskolin-mediated (FSK, n=2) and NaF-stimulated (NaF, n=2) adenylyl cyclase activity in CHW cells expressing 5 pmol β_2AR/mg protein. Data are expressed as pmol cAMP produced per mg membrane protein per minute ± SEM. Statistical significance of the difference are indicated b an astrisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. Membranes were treated with either vhicle (lane 1) or the TM VI peptide at a concentration of 0.15 ug/ul (lane 2) for 30 minutes at room temperature. Membranes from untransfected CHW cells had no detectable receptors (data not shown). **B,** Effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in mouse Ltk⁻ cells. Membranes were prepared from Ltk⁻ cells stably expressing 200 fmol of human β_2AR/mg membrane protein. Isoproterenol-stimulated adenylyl cyclase activity was then assessed in membranes treated with vehicle (open circles), TM VI peptide (closed squares), control peptide TM VI Ala (closed circles), or the D2 TM VII control peptide (open triangles). Data are expressed relative to the maximal stimulation obtained with vehicle treated membranes and represent mean ± SEM or 3 independent exeriments. Peptides were used at a concentration of 0.15 ug/ul.

DETAILED DESCRIPTION OF THE INVENTION

The following common abbreviations are used throughout the specification and in the claims:

The abbreviation. IP is inositol phosphate.

The abbreviation BASED is bis [β-(4 azidosalicylamindo) ethyl] disulphide

The abbreviation. 5-HT is 5-hydroxytryptamine.

The abbreviation, DOI is 2,5-dimethoxy-4-iodoamphetamine hydrobromide.

The abbreviation, PBS is phosphate buffered saline.

The abbreviation, β_2AR is β_2-adrenergic receptor.

The abbreviation, GPCR is G protein-coupled receptor.

The abbreviation, GpA is glycophorin A.

The abbreviation. HA is influenza hemagglutinin.

The abbreviation TM VI is transmembrane domain 6.

The abbreviation, NDI is nephrogenic diabetes insipidus.

The term "any amino acid" as used herein includes the L-isomers and D-isomers of the naturally occurring amino acids, as well as other "non-protein" α-amino acids commonly utilized by those in the peptide chemistry arts when preparing synthetic analogues of naturally occurring peptides. The naturally occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine,. threonine, phenylalanine, tyrosine. trptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine. Y-carboxyglutamic acid, arginine, ornithine and lysine. Examples of "non-protein" α-amino acids include norleucine. norvaline, alloisoleucine. homoarginine, thioproline. dehydroproline, hydroxyproline (Hyp), isonipecotic acid (Inp), homoserine. cyclohexylglycine (Chg), α-amino-n-butyric acid (Aba), cyclohexylalanine (Cha). aminophenylgutyric acid (Pba). phenylalanines substituted at the ortho, meta, or para

position of the phenyl moiety with one or two of the following: a (C_1-C_4) alkyl, a (C_1-C_4) alkoxy, halogen or nitro groups or substitute with a mthylenedioxy group, β-2- and 3-thienylalanine, β-2- and 3-furanylalanine, β-2-, and 3- and 4-pyridylalanine, β-(benzothienyl-2- and 3-yl)alanine, β-(1- and 2-napthyl)alanine, O-alkylated derivatives of serine, threonine or tyrosine, S-alkylated cysteine, S-alkylated homocysteine, O-sulfate, O-phosphate and O-carboxylate esters of tryosine, 3-sulfo-tyrosine, 3-carboxy-tyrosine, 3-phospho-tyrosine, 4-methane sulfonic acid ester of tyrosine, 4-methane phosphonic acid ester of tyrosine, 3,5-diiodotyrosine, 3-nitrotyrosine, ε-alkyl lysine, delta-alkyl ornithine, and the ε-isomers of any of the above amino acids. Unless specifically indicated, all amino acids referred to in this application are in the L-form.

The term "positively charged amino acid" as used in this application includes any naturally occurring or non-naturally occurring amino acid having a positively charged side chain. Examples of positively charged amino acids are arginine, lysine, histidine, homoarginine, ornithine and delta-alkyl ornithine.

The term "amino acid containing an aryl side chain" as used herein means any amino acid having an aromatic group. Tyrosine, phenylalanine, tryptophan, O-sulfate esters of tyrosine and 5-nitrotyrosine exemplify such amino acids.

The term "polar amino acid" means any amino acid having an uncharged side chain which is relatively soluble in water. Examples include glutamine, asparagine, glycine, serine, hydroxyproline and homoserine.

The term "hydrophobic amino acid" mans any amino acid having an uncharged side chain which is relativly insoluble in water. This group includes leucine, valine, tryptophan, norleucine, norvaline, alloisoleucine, thioproline, dehydroproline, cyclohexylalanine and cyclohexylglycine.

The term "patient" as used in this application refers to any mammal, especially humans.

The term "backbone chain" as used herein, refers to the portion of a chemical structure that defines the smallest number of consecutive bonds that can be traced from one end of that chemical structure to the other. The atomic components that make up a backbone chain may comprise any atoms that are capable of forming bonds with at least two other atoms.

The term "peptide-lead" as used herein, refers to the underivitized peptide that is modelled on one of the transmembrane domains in the first step of the design of the compounds of this invention.

The term "parent GPCR" as used herein, refers to the GPCR from which the peptide-lead is derived. Molecules of this invention are designed using the peptide-lead as the model for antagonists that are selectively inhibitory towards the parent GPCR or closely related receptors.

The terms "selective inhibition" and "selectively inhibits" as used herein, refers to the ability of the peptide to inhibit the actions of a species of GPCR and/or closely related

receptors without affecting the activity of other non-related species of receptors to any significant extent. Significant extent means there must be at least a 10-fold magnitude difference in inhibitory activies between the parent receptor and other types of receptors.

The present invention relates to novel peptides and/or peptidomimetic compounds that are modeled on the transmembrane region of G protein-coupled receptors. The peptide and peptidergic molecules of this invention selectively bind to the GPCR from which they were designed. This property allows the molecules of this invention to inhibit those functions of the GPCR that are mediated through this receptor.

Applicant has discovered that peptides corresponding to residues 276-296, residing in the 6th transmembrane region of the β_2-adrenergic receptor, selectively inhibits agonist promoted stimulation of adenylyl cyclase activity. Applicant believes that this peptide forms structural complimentarity with the receptor from which it was derived, preventing the receptor from interacting with the molecules necessary to effect its activities. Peptides and other molecules which comprise such features will bind to the receptor from which the peptide was derived and inhibi its activation in vivo and in vitro. These peptides and other molecules, as well as compositions and methods which employ them, make up the present invention.

According to one embodiment, the GPCR antagonists of the present invention are characterized by complimentarity to one of the transmembrane domains of the parent receptor. Preferably, that region is modelled on one of the transmembrane domains. The

antagonists of this invention are further characterized by: (1) the ability to inhibit the activity of the parent GPCR and possibly closely related receptors. and (2) the inability to significantly inhibit other types of receptors (i.e. other receptors can be inhibited if there is a 10-fold magnitude difference in activities)

Determination of Candidate Transmembrane Sequences (TMS)

In order to identify and define the peptide-lead that will serve as a model for design and synthesis of antagonists of this invention, one of ordinary skill in the art begins by synthesizing a peptide consisting of amino acids corresponding to one of the transmembrane membrane domains of the GPCR of interest. This peptide itself can potentially be a GPCR antagonist of the present invention.

Each transmembrane sequence provides a potential model for a peptide-lead that will be used to design peptides or peptidiomimetic compounds that will function as a selective antagonist against the same GPCR (parent-GPCR) and possibly closely related receptors. Thus, the first step is to identify the transmembrane domain of the receptor of interest. There are a number of techniques well known in the art available for determining transmembrane regions of the GPCR. These include hydropathy plots to identify the hydrophobic segments. The secondary structure can also be analyzed to identify alpha-helix structures. This information can be used in combination with the general view that GPCRs are organized in a manner similar to bacteriorhodopsin, which has been shown to possess seven transmembrane alpha-helical hydrophobic regions.

Computer-assisted structural assessment tools than can be used to examine the hydropathy (Kyte. J.. and Doolittle, R.F., *J. Mol. Biol.*, 157:105-132, 1982), flexibility (Karplus, P.A., and Schultz. G.E., *Naturwissenschaften*, 72:212-213, 1985), and secondary structure (Chou. P.Y., and Fasman,G.D., *Annu. Rev. Biochem.*, 47:251-276, 1976) of the trans-membrane domains are well known in the art (for eg., see: Krystek et al., *Endocrinology* (Baltimore) 117:1125-1131, 1985a; *Endocrinology* (Baltimore) 117:1110-1124, 1985b). Surface profiles (eg. the Kyte and Doolittle scale) can be used to identify regions of the G protein-coupled receptor which should be accessible for protein-protein interactions, with the caveat that such analysis does not take into account the carbohydrate chains located on proteins, and disulfide bonds are not adequately analyzed by these methods. Correlation of flexibility plots with homology plots and surface profiles may be helpful in identifying specific regions of protein structure. The most flexible regions of a protein are reportedly associated with protein binding sites (Richardson, ,1981; Van Regenmortel, , 1987). Flexibility plots ostensibly identify the most flexible regions of a polypeptide but do not take into account disulfide bonding (Spinella et al., 1989) Since disulfides are known to stabilize protein structures (Richardson,1981), and the flexibility of a molecule that is highly disulfide bonded is probably overestimated by this technique, this limitation should be kept in mind.

The potential activities of this peptide is tested in the Selective Antagonist Assay described below.

Design and Synthesis of Peptides Modeled on TMS

In designing an antagonist molecule according to this invention,two important considerations must be taken into account. First, the molecule must be able to physically associate with the parent-GPCR. The present theory of peptide binding suggests that the initial step in binding requires, at a minimum, an ionic interaction between the receptor and the peptide. It is also probable that other molecular interactions, such as hydrogen bonding and hydrophobic interactions, are important for this association. Therefore, the identification and maintenance of these interactions are critical in designing a potent GPCR antagonist.

The second consideration in designing the antagonists of this invention is secondary and tertiary structure. While certain portions of the antagonist do not directly participate in molecular interactions with the receptor, they may play a role in the overall conformation of the antagonist. This, in turn, can have a dramatic effect on potency. If the antagonist cannot assume the proper conformation, the molecular interactions required for association with the receptor cannot be achieved, even if the components capable of forming such interactions are present in the molecule. Accordingly, an antagonist of this invention must be designed so that it assumes a conformation which allows it to associate with the receptor. conformational requirements may be in the nature of overall three-dimensional structure and orientation of the antagonist, or merely the spacing between two sites on the antagonist which directly interact with the receptor.

To test which of the amino acids in the 15-20 amino acid peptide-lead are responsible for crucial molecular interactions with the parent GPCR, a simple alanine scanning procedure is carried out. In this procedure, a series of peptides, each having a single alanine substitution at a different residue, is synthesized. The peptides are then assayed to determine if they inhibit selective GPCR antagonist activity.

Those alanine-substituted peptides which retain a GPCR antagonist activity, indicate portions of the antagonist that do not directly interact with the receptor and which do not have side chains which play a critical role in the folding of the antagonist molecule. Such peptides are preferred antagonists of the present invention. Conversely, those peptides which lack or have greatly reduced antagonist activity point out areas of the antagonist that are important for activity. These latter peptides suggest the nature of an important interior intramolecular interaction based upon the amino acid substituted for. For example, an argining-to-alanine substitution which resulted in reduced activity suggests the location of an important positive charge - either an ionic interaction with the receptor or an intramolecular ionic interaction within the antagonist - which is required to maintain optimal conformation. A serine-to-alanine substitution which had a negative effect on activity indicates the location of an important hydrogen bond. Again, the hydrogen bond may be between the antagonist and the GPCR, or it may be an intramolecular hydrogen bond that plays an important role in the conformation of the antagonist.

Those of skill in the art will realize that distinguishing between whether a structural feature is important for an inter- or intramolecular interaction can only be achieved by examining

an X-ray crystal structure of the antagonist-receptor complex. However, that distinction is of little import in designing th antagonists of this invention. Once the nature of the interaction is determined, i.e., electrostatic, hydrophobic, ionic, the choice of potential substitutes at that position becomes clear.

To further ascertain those sites that are important for proper folding and orientation of the GPCR antagonists of this invention, a single poisition deletion analysis is performed. In this procedure, a series of peptides containing single deletions at positions which do not affect inhibitor activity (as determined above) are synthesized and assayed for antagonist activity. The peptides from this series that retain significant antagonist activity indicate areas of the antagonist that are not essential for proper conformation. Such peptides are also included within the scope of this invention.

Deletion peptides from this series which have significantly lower antagonist activity indicate the location of components which provide critical spacing in the antagonist. This may be verified by replacing the deleted amino acid with a different, yet analogous structure. For example, substitution of any conformationally important amino acid with a three carbon alkyl chain without a significant loss of activity confirms that spacing is critical at that part of the molecule.

Additional information about important structural and conformational features necessary for designing a potent GPCR antagonist of this invention may be obtained through 3-dimensional X-ray crystallographic procedures coupled with computer modelling.

Specifically, one of ordinary skill in the art may analyze a GPCR/peptide-lead using such a method. Alternatively, one of average skill in the art could employ multiple alanine substitutions or multiple deletions to identify important intramolecular interactions in the antagonist itself. It will also be aparent that each new GPCR antagonist designed and tested will, itself, provide additional information about structural features important for GPCR inhibition.

Once the critical residues in the peptide-lead have been located and characterized, other GPCR antagonists of this invention may be designed and synthesized. This is achieved by substituting the identified key residues of the GPCR peptide antagonist with other components having similar features. These substitutions will initially be conservative, i.e., the replacement component will have approximately the same size, shape, hydrophobicity and charge as the key residue. Those of ordinary skill in the art are well aware of appropriate replacements for a given amino acid [Dayhoff et al., in *Atlas of Protein Sequence and Structure No. 5*, 1978 and Argos et al., *EMBO J.*, 8, pp. 779-85 (1989)]. Typical conservative substitutions for an amino acid are other amino acids with similar charges. for example, aspartic acid for glutamic acid, arginine for lysine, asparagine for glutamine, hydroxyproline for proline and *vice versa*. Substitutions with non-natural amino acids may also be performed to reduce the peptidic nature of the antagonist. Some examples are cyclohexylalanine for tyrosine, sarcosine for glycine, statine for threonine and homoarginine for arginine. These modifications may increase the biological stability of the antagonist, in addition to increasing its potency.

After the molecule containing the substitute component is shown to be an effective GPCR antagonist. less conservative replacements may be made at the same position. These substitutions typically involve the introduction of non-amino acid components which contain the important feature imparted by the amino acid at that position. Such substitutes are well-known in the art. For example, the sequence Leu-Val-Arg can be replaced by p-guanidinobenzoic acid. This substitution maintains the hydrophobicity of Leu-Val. as well as the guanidinium functionality of Arg.

It will be apparent that there is greater freedom in selecting the substitute for a non-essential amino acid in the peptide-lead sequence. Moreover, a non-essential amino acid may simply be eliminated. Almost any substitute that does not impart a change in conformation may be employed for a nonessential amino acid. These include, but are not limited to, straight chain alkyl and acyl groups. Also, because of the importance of the net positive charge of the antagonist, anionic substitutes should be avoided. Components which are known in the art to alter conformation should also be avoided. One such component is proline, an amino acid which causes a turn structure in a molecule. Others are well-known in the art [G.D. Rose et al., "Turns in Peptides and Proteins", Adv. Prot. Chem., 37, pp. 1-110 (1985)].

In addition to those antagonists resulting from the substitutions and deletions described above. novel GPCR antagonists according to this invention may be designed by insertions at various sites along the peptide-lead. To determine areas of the peptide-lead where a component may potentially be inserted, a series of peptides having a single alanine insertion at various sites is synthesized. Those peptides from this series which retain antagonist

activity indicate potential insertion sites.

In choosing a component to be inserted, one should be guided by the same considerations set forth above in selecting a substitute component. Specifically, one must keep in mind how the insertions may potentially affect the molecular interactions between the antagonist and the GPCR and how they affect conformation of the antagonist. For example, the insertion of an anionic componnt adjacent to a critical cationic amino acid in the peptide-lead could interfere with an important ionic interaction and should therefore be avoided. Similarly, the insertion of a component which is known to cause structural perturbations, e.g., a proline, should also be avoided.

Using any or all of the above deletion, substitution and insertion techniques allows those of ordinary skill in the art to design GPCR peptide antagonists according to this invention. Moreover, the potential effect of any of these changes may be theoretically observed prior to synthesizing the antagonist through the use of computer modelling techniques known in the art. Such modelling allows one to observe the predicted structure of a GPCR complexed with the potential antagonist. If that theoretical structure suggests insufficient interaction between the receptor and the potential antagonist, one need not spend time and resources in synthesizing and testing the molecule. On the other hand, if computer modelling indicates a strong interaction, the molecule may then be synthesized and assayed for antagonist activity. In this manner, inoperative molecules may be eliminated before they are synthesized.

Finaly, cyclic derivatives of any antagonist designed by the above techniques are also part of the present invention. Cyclization may allow the antagonist to assume a more favorable conformation for association with the GPCR. Cyclization may be achieved by methods well-known to those in the art. One method is the formation of a disulfide bond between two non-adjacent cysteine residues (D- or L-conformation) or any two appropriately spaced components having free sulfhydryl groups. It will be understood that disulfide bonds, as well as other intramolecular covalent bonds, may be formed between a variety of components within the antagonist. The components which form such bonds may be side chains of amino acids, non-amino acid components or a combination of the two.

The most preferred antagonists of the present invention are modelled after the peptide-lead which comprises the formula: SEQ ID NO: 1: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH. In order to ensure the entire predicted region be contained in a single peptide, it may be wise to extend both the amino terminus and the carboxyl terminus.

The synthesis of the peptides of this invention including derivation, activation, and coupling of protected amino acid residues, and their purification, and the analytical methods for determining identity and purity are included in the general body of knowledge of peptide chemistry, as described in Houben Weyl *Methoden der Organischen Chemie,* (1974), Vol. 16, parts I & II for solution-phase synthesis, and in *Solid Phase Peptide Synthesis*, (1984), by Stewart and Young for synthesis by the solid-phase method of Merrifield.

Any chemist skilled in the art of peptide synthesis can synthesize the GPCR peptide

antagonists of this invention by standard solution methods. These include enzymatic cleavage of GPCR, recombinant DNA techniques, solid-phase peptide synthesis, solution-phase peptide synthesis, organic chemical synthesis techniques, or a combination of these techniques. The choice of synthesis technique will, of course, depend upon the composition of the particular antagonist. In a preferred embodiment of this invention, the GPCR antagonist is entirely peptidic and is synthesized by solid-phase peptide synthesis techniques, solution-phase peptide synthesis techniques or a combination thereof which constitute the most cost-efficient procedures for producing commercial quantities of these antagonists.

When "non-protein" amino acids are contained in the GPCR antagonist, they may be either added directly to the growing chain during peptide synthesis or prepared by chemical modification of the complete synthesized peptide, depending on the nature of the desired "non-protein" amino acid. Those of skill in the chemical synthesis art are well aware of which "non-protein" amino acids may be added directly and which must be synthesized by chemically modifying the complete peptide chain following peptide synthesis.

The synthesis of those GPCR antagonists of this invention which contain both non-amino acid and peptidic portions is preferably achieved by a mixed heterologous/solid phase technique. This technique involves the solid-phase synthesis of all or most of the peptide portion of the molecule. This is followed by the addition of the non-amino acid components which are synthesized by solution phase techniques and then coupled to the peptidic portion via solid-phase or solution-phase methods. Any remaining peptidic portions may then be added via solid-phase or solution-phase methods.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various n-protecting groups. e.g., the carbobenzyloxy group or the t-butyloxycarbonyl group (BOC), various coupling reagents. e.g., dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g., esters of N-hydroxypthalimide or N-hydroxy-succinimide, and the various cleavage reagents, e.g., trifluoracetic acid (TFA), HCl in dioxane, boron tris-(trifluoracetate) and cyanogen bromide, and reaction in solution with isolation and purification of intermediates is well-known classical peptide methodology.

A preferred peptide synthesis method follows conventional Merrifield solid-phase procedures. See Merrifield, *J. Amer. Chem. Soc.* 85, 2149-54 (1963) and *Science* 150, 178-85 (1965). This procedure, though using many of the same chemical reactions and blocking groups of classical peptide synthesis, provides a growing peptide chain anchored by its carboxy terminus to a solid support, usually cross-linked polystyrene, styrenedivinylbenzene copolymer or, preferably, p-methylbenzhydrylamine polymer for synthesizing peptide amides. This method conveniently simplifies the number of procedural manipulations since removal of the excess reagents at each step is effected simply by washing the polymer.

Further background information on the established solid phase synthesis procedure can be had by reference to the treatise by Stewart and Young, *"Solid Phase Peptide Synthesis,"* W.H. Freeman 7 Co., San Francisco, 1969, and the review chapter by Merrifield in

Advances in Enzymology 32, pp. 221-296, F.F. Nold. Ed., Interscience Publishers, New York, 1969; and Erickson and Merrifield, *The Proteins*, Vol 2, p. 255 et seq. (ed. Neurath and Hill), Academic Press, New York, 1976.

Design of Peptidomimetic Compounds

It is well known in the drug design art to look for a substitute compound that mimics the conformation and desirable features of a particular peptide, e.g., an oligopeptide, once such peptide has been found, but that avoids the undesirable features of a peptide compound, e.g., flexibility (loss of conformation) and bond breakdown. Such a compound that mimics a peptide is known as a "peptidomimetic". There are a number of methods for designing peptidomimetic compounds that are known in the art.

In each case, the starting point for designing a peptidomimetic compound is the sequence and/or conformation of a particular oligopeptide or peptide of interest. For example, see, Spatola, A.F. *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins* (Weistein, B, Ed.), Vol. 7, pp. 267-357, Marcel Dekker, New York (1983), which describes the use of methylenethio bioisostere [CH_2S] as an amide replacement in enkephalin analogues; and Szelke et al., *In Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium,* (Hruby and Rich, Eds.); pp. 579-582, Pierce Chemical Co., Rockford, Ill. (1983), which describes renin inhibitors having both teh methyleneamino [CH_2NH] and hydroxyethylene [$CHOHCH_2$] bioisosteres at the Leu-Val amide bond in the 6-18 octapeptide derived from angiotensinogen.

It is also known in the art to use computer simulation in an attempt to predict a stable conformation of a peptide. That is. because a peptide is a sequence of amino acid residues, each containing known atoms bonded together in known molecules having known bonding lengths. with known electrostatic properties associated with each atom. it is possible to simulate a peptide structure on the computer. An example of such a compuer based method of rational drug design that identifies bioactive peptidomimetics that can be effectively used as drugs is U.S. Patent No. 5,331,573. This method predicts the most probable secondary and/or tertiary structures of a polypeptide without any presumptions as to the conformation of the underlying primary or secondary structure. The method involves computer simulation of the peptide in a manner that simulates a real-size primary structure in an aqueous environment, shrinking the size of the polypeptide isobarically and isothermally, and expanding the simulated polypeptide to its real size in selected time periods. A useful set of tools, terms Balaji plots, energy conformational maps and probability maps, assist in identifying those portions of the predicted peptide structure that are most flexible or most rigid. The overall method of this technique involves the following steps:

(a) simulating the most probable conformations of a given polypeptide;

(b) selecting the most probable conformation of the peptides thus simulated;

(c) designing and synthesizing a chemically modified analog of the selected peptide;

(d) evaluating the bioactivity of the synthesized chemically modified analog of the selected peptide; and thereafter, optionally

(e) designing a suitable peptidomimetic based on the conformation of the synthesized chemically modified analog of the selected peptide.

In carrying out this method, if it is noted that the chemically modified analog of the selected peptide is not bioactive, as determined through suitable testing, then an additional step relates to determining whether other chemically modified analogs should be designed for this same selected peptide. If so, then another chemically modified analog for the selected peptide is designed and the bioactivity of this newly designed chemically modified analog is evaluated. If a determination is made that another chemically modified analog for this same peptide should not be designed, then the nest most probable conformation of the simulated peptide is selected and a chemicaly modified analog is designed and synthesized for such selected peptide and the process is repeated.

Experimental Assessment of Candidate Peptides

The ability of these peptides to function as selective antagonists against the receptor from which they have been derived and/or closely related receptors, can be measured using one of the many techniques available in the art. These assay procedures are performed using preparations, such as tissues, tissue extracts, cells or extracts thereof, containing the specific GPCR of interest, which have been made to express defined cloned GPCRs using techniques familiar to those skilled in the art.

Test assays measure the consquence of receptor activity on other cellular components involved in the signalling pathway(s) controlled by receptor. These measurements can be performed at the level of G protein activation by a GPCR. For example, the rate of binding of guanyl neucleotide analogue GTPgS35 or hydrolysis of GTP by activated G protein can

be measured. These types of measurements may also be made by measuring the activity of effector enzymes such as adenyl cyclase activation (Gs-coupled receptors) and inhibition (Gi-coupled receptors), which are consequences of activation of respective subsets of GPCRs.

These parameters can be measured in whole cells by quantitating cAMP produced in cells during a specified period. Alternatively, membrane fragments from cells can be used for assay by measuring conversion of radioactive ATP to cAMP by adenlyl cyclase. Receptor activation of Gq-coupled receptors can be measured in intact cells by production of inositol phosphates from diacylglycerol through stimulation of phospholipase C activity. Downstream events can also be measured to monitor receptor activity including: protein kinase A activity (which is under control of cAMP levels) for Gs and Gi-coupled receptors; intracellular calcium concentrations can be measured using specific fluorscent probes (e.g. fura-2) for Gq receptors; activation or inhibition of transcription of specific genes under control of receptor activity, can be measured either directly (e.g. measure mRNA levels) or by reporter gene assays which replace natural gene with readily measured marker enzyme (e.g. beta-galactosidase, luciferase). Finally, receptor activity can be assayed by measuring cellular and tissue responses to receptor activation, such as muscle contraction (classical bioassay on smooth muscle strips), cell membrane potential (e.g. neurons), secretion (glandular cells), cell proliferation, etc.

The specifics of peptide-assessment assays would thus involve the following steps:
Adding aqueous solution containing peptide, derivative, or peptidiomimetic compound to

be tested to solution containing a GPCR preparation (tissue, cell or extract); adding agonist to the same solution; measuring the response to agonist by means of assay as described above; comparing the magnitude of the response to agonist in presence of the peptide or peptidiomimetic compound to that in absence of test molecule under otherwise identical conditions. Decrease in agonist-induced response in the presence of peptide or peptidiomimetic compound indicates antagonist activity.

Antagonist can be further characterized by testing: varying concentrations of peptide with fixed concentration of peptide or peptidiomimetic compound with fixed concentration agonist (to determine the potency of the antagonist compound) and then varying the concentration of the agonist with fixed peptide concentration (to determine competitive vs. non-competitive action). Finally, measuring the effect of peptide or peptidiomimetic compound on distantly-related receptor can be performed to determine selectivity.

Antagonist activity of peptide or peptidiomimetic compounds can also be assessed by measuring the antagonists affect on spontaneous receptor activity (i.e., basal activity in absence of added agonist). In this case, the same assay systems can be used without agonist and look for decrease in receptor activity in presence of peptide.

All assays described here are familar to those versed in the art, and described in detail in numerous scientific publications and methods manuals.

Use of Peptides as Drugs

The present invention also provides a method for treatment of G protein-coupled receptor mediated disease in patients, such as mammals, including humans, which comprises the step of administering to the patient a pharmaceutically effective amount of a compound, a pharmaceutically acceptable salt thereof, or a pharmaceutical composition as described.

The present invention also provides pharmaceutical compositions which comprise a pharmaceutically effective amount of the peptides or peptidomimetic compounds of this invention, or pharmaceutically acceptable salts thereof, and, preferably, a pharmaceutically acceptable carrier or adjuvant. Therapeutic methods of this invention comprise the step of treating patients in a pharmaceutically acceptable manner with those compounds or compositions. Such compositions may be in the form of tablets, capsules, caplets, powders, granules, lozenges, suppositories, reconstitutable powders, or liquid preparations, such as oral or sterile parenteral solutions or suspensions.

The therapeutic agents of the present invention may be administered alone or in combination with pharmaceutically acceptable carriers. The proportion of each carrier is determined by the solubility and chemical nature of the compound, the route of administration, and standard pharmaceutical practice.

In order to obtain consistency of administration, it is preferred that a composition of the invention is in the form of a unit dose. The unit dose presentation forms for oral administration may be tablets and capsules and may contain conventional excipients. For example, binding agents, such as acacia, gelatin, sorbitol, or polyvinylpyrolidone; fillers,

such as lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants such as magnesium stearate; disintegrants, such as starch, polyvinylpyrrolidone, sodium starch glycollate or microcrystalline cellulose; or pharmaceutically acceptable wetting agents such as sodium lauryl sulphate.

The compounds may be injected parenterally; this being intramuscularly, intravenously, or subcutaneously. For parenternal administration, the compound may be used in the form of sterile solutions containing other solutes, for example, sufficient saline or glucose to make the solution isotonic.

The compounds may be administered orally in the form of tablets, capsules, or granules containing suitable excipients such as starch, lactose, white sugar and the like. The compounds may be administered orally in the form of solutions which may contain colouring and/or flavouring agents. The compounds may also be administered sublingually in the form of traches or lozenges in which each active ingredient is mixed with sugar or corn syrups, flavouring agents and dyes, and then dehydrated sufficiently to make the mixture suitable for pressing into solid form.

The solid oral compositions may be prepared by conventional methods of blending, filling, tabletting, or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. The tablets may be coated according to methods well known in normal pharmaceutical practice, in particular with an enteric coating.

Oral liquid preparations may be in the form of emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may or may not contain conventional additives. For example suspending agents, such as sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminium stearate gel, or hydrogenated edible fats; emulsifying agents, such as sorbitan monooleate or acacia; non-aqueous vehicles (which may include edible oils), such as almond oil, fractionated coconut oil, oily esters selected from the group consisting of lycerine, propylene glycol, ethylene glycol, and ethyl alcohol; preservatives, for instance metyl para-hydroxybenzoate, ethyl para-hydroxybenzoate, n-propyl parahydroxybenzoate, or n-butyl parahydroxybenzoate of sorbic acid; and, if desired, conventional flavoring or coloring agents.

For parenternal administration, fluid unit dosage forms may be prepared by utilizing the peptide and a sterile vehicle, and, depending on the concentration employed, may be either suspended or dissolved in the vehicle. Once in solution, the compound may be injected and filter sterilized before filling a suitable vial or ampoule and subsequently sealing the carrier or storage package. Adjuvants, such as a local anaesthetic, a preservative or a buffering agent, may be dissolved in the vehicle prior to use. Stability of the pharmaceutical composition may be enhanced by freezing the composition after filling the vial and removing the water under vacuum, (e.g., freeze drying the composition). Parenteral suspensions may be prepared in substantially the same manner, except that the peptide should be suspended in the vehicle rather than being dissolved, and, further, sterilization is not achievable by filtration. The compound may be sterilized, however, by exposing it

to ethylene oxide before suspending it in the sterile vehicle. A surfactant or wetting solution may be advantageously included in the composition to facilitate uniform distribution of the compound.

The pharmaceutical compositions of this invention comprise a pharmaceutically effective amount of a compound of this invention and a pharmaceutically acceptable carrier. Typically, they contain from about 0.1% to about 99% by weight, preferably from about 10% to about 60% by weight, of a compound of this invention, depending on which method of administration is employed.

Physicians will determine the dosage of the present therapeutic agents which will be most suitable. Dosages may vary with the mode of administration and the particular peptide or peptidomimtic compound chosen. In addition, the dosage may vary with the particular patient under treatment. The dosage of the compound used in the treatment will vary, depending on the seriousness of the disorder, the weight of the patient, the relative efficacy of the compound and the judgment of the treating physician. Such therapy may extend for several weeks, in an intermittent or uninterrupted manner, until the patient's symptoms are elimінted.

It is appreciated that the compounds of the present invention can be modified by one skilled in the art in such a manner as to prevent access into the central nervous system such that they can function in peripheral tissues to affect peripheral G protein coupled receptor mediated events.

To further assist in understanding the present invention, the following non-limiting examples of such peptides are provided. The following examples, of course, should not be construed as specifically limiting the present invention, variations presently known or later developed, which would be within the purview of one skilled in the art and considered to fall within the scope of the present invention as described herein.

EXAMPLES

Effects of TM VI Peptide on β_2AR Stimulated Adenylyl Cyclase Activity in Sf9 Cells.

Membranes from Sf9 and mammalian cells infected with recombinant baculoviruses, encoding the wildtype human β_2-adrenergic receptor, were used to determine the effect of various peptides on the ability of the β_2AR to stimulate adenylyl cyclase activity.

Synthesis of peptides

Peptides were synthesized on solid-phase supports using f-moc chemistry (40) on a BioLynx 4175 manual peptide synthesizer (LKB). Peptides were solubilized in the following buffer: 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA (plus the protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5 mg/ml soybean trypsin inhibitor), 0.05% digitonin and 10% DMSO. Peptide sequences were conformed either by mass spectrometry or amino acid analysis.

Peptides were synthesized based on: 1) the sixth transmembrane region of the β_2AR, referred to as β_2AR TM VI peptides consisting of residues 276-296: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH, 2) a second peptide with Ala residues substituted

at positions 276, 280, and 284 NH_2-AIIMATFTACWLPFFIVNIVH-COOH, 3) a peptide derived from residues 407-426 of the D2 dopamine receptor corresponding to the seventh transmembrane region of the receptor - referred to as D2 TM VII NH_2YIIPNVASNVYGLWTFASYL-COOH, 4) a peptide derived from the C-terminal tail of the β_2AR consisting of residues 347-358 NH_2 LKAYGNGYSSNG-COOH and 5) an additional peptide unrelated to β_2AR (but of similar size as the TM VI peptide) corresponding to the sequence NH_2-SIQHLSTGHDHDDVDVGEQQ-COOH.

Assessment of Antagonistic Activities of Synthetic Peptides

To assess the effect of the different peptides or β_2AR ligands on the relative amount of different receptor species the following experiments were performed.

Mammalian or Sf9 cells were infected with recombinant baculoviruses, encoding the wildtype human β_2-adrenergic receptor, constructed as described (33). Cells were infected with recombinant baculoviruses at multiplicities of infection ranging from 3 - 5.

Sf9 Cell Culture

Sf9 cells were maintained at 27°C in serum-supplemented [10% fetal bovine serum (FBS) v/v] Grace's insect medium (Gibco-BRL) with gentamycin and fungizone. Cells were grown either as monolayers in T flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell taring due to agitation. Cells were infected at log phase at a densit of 1 x 10^6 cells per ml for 48 h.

Mammalian Cell Culture

CHW and LTK cell lines with and without stably transfected β_2AR were maintained as described (34). Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamate, 10% FBS, gentamycin and fungizone. Transfected CHW cells expressed -5 pmol receptor/mg protein while transfected LTK cells expressed 200 fmol receptor/mg protein. Stably transfected cell lines were grown in the presence of 150 ug/ml G418.

For transient expression of V2 vasopressin receptors the following prodecures were followed. COS-7 cells were maintained in supplemented DMEM as described above. Genomic DNA for the V2 vasopressin receptor was isolated from nephrogenic diabetes insipidus (NDI) patients or unaffected individuals, subclosed into a construct containing a c-*myc* epitope tag and ligated into a mammalian expression vector, pBC12BI (35). Using DEAE-dextran, COS-7 cells were transiently transfected with the expression vector encoding either wildtype V2 vasopressin receptor, a truncation mutant O-11 or with vector alone for 48 hours.

Membrane Preparation

Membranes were prepared as follows and washed. Sf9 or mammalian cells were washed twice with ice-cold PBS. The cells were then disrupted by homogenization with a polytron in 10 ml of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus a protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5 mg/ml soybean trypsin inhibitor). Lysates were centrifuged at 500 x *g* for 5 minutes at 4°C.

the pellets homogenized as before, spun again and the supernatants were pooled. The supernatant was then centrifuged at 45,000 x *g* for 20 minutes and the pellets washed twice in the same buffer.

Assessment of Peptide Activity

To assess the effect of the different peptides on the β_2AR expressed in Sf9 and mammalian cells, the following experiments were performed. Generally, membrane preparations from mammalian or Sf9 cells infected with recombinant bacculovirus expressing human β_2AR were treated with increasing concentrations of the different peptides at room temperatures and for various times as indicated below. Peptide antagonist activity was assessed by assaying adenylyl cyclase activity. Membrane adenylyl cyclase activity was assayed as previously described (Mouillac, B., et al., *J. Biol. Chem.*, 267:21733-21737, 1992; Salomon, Y et al., *Anal. Biochem.*, 58:541-548, 1974). The results of these sets of experiments are presented in Figure 1.

Membranes were prepared and washed as described above. Again 10 uL of membranes (3-5 ug of protein) were used in a total volume of 50 uL. In some experiments, the peptides or the buffer used to solubilize them were added to the enzyme assay mix. Enzyme activities were determined in the presence of nM to 100 uM isoproterenol, 100 uM forskolin or 10 mM NaF. Data were calculated as pmoles cAMP produced/min/mg protein and were analyzed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

As shown in Figure 1, the addition of TM VI peptide to membrane preparations at a

concentration of 0.15 ug/ul significantly reduced isoproterenol-stimulated adenyl cyclase activity ($p<0.05$). In contrast. neither the peptide solubilization buffer (data not shown) nor control peptides (β_2AR TM VI-Ala or D2 TM VII) had significant effects on isoproterenol-stimulated adenylyl cyclase activity.

The effect of the peptide was receptor-specific as it had no effect on either NaF-mediated or forskolin-mediated adenylyl cyclase stimulation (Figure 1b). Notably, the ligand-independent basal adenylyl cyclase activity was slightly inhibited by the TM VI peptide suggesting that it may effect the spontaneous activity of the receptor as well. Indeed, spontaneous receptor activity is in large part responsible for the ligand-independent adenylyl cyclase activity observed in both Sf9 and mammalian cells expressing β_2AR (71). A receptor-dependent effect is also supported b the fact that the TM VI peptide was without effect on basal cyclase activity in Sf9 cells which were infected with the wildtype baculovirus (data not shown). Also consistent with a receptor-specific action of the peptide is the observation that D1 dopamine receptor-stimulated adenylyl cyclase activity was not significantly affected by the TM VI peptide (Figure 1c). Moreover, the inhibitory action of the TM VI peptide on receptor-mediated adenylyl cyclase activity was dose-dependent (Figure 1c).

The effect of TM VI peptide on adenylyl cclase stimulation does not result from a loss of receptor sites as neither the affinity or the maximum number of binding sites for ^{125}-I CYP were affected ($K_D=1.8 \pm 0.5 \times 10^{-10}$ and $B_{mas} = 16.5 \pm 2$ pmol/mg protein for untreated membranes compared with $K_D=4.2 \pm 1.5 \times 10^{-10}$ and $B_{mas} = 21.3 \pm 4.5$ pmol/mg protein

for TM VI peptide treated membranes, n=3 for both determinations).

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Consequently, such changes and modifications are properly, equitably, and "intended" to be, within the full range of equivalence of the following claims.

Claims:

1. A compound comprising a peptide, analog, fragment or derivative thereof which is characterized as being:
a) 5 - 30 amino acid residues in length;
b) significantly homologous to a transmembrane domain of a G protein-coupled receptor; and
c) selectively inhibits the activity of a G protein-coupled receptor.

2. A peptidomimetic compound modeled on a peptide, analog, fragment or derivative thereof which is characterized as being:
a) 5 - 30 amino acid residues in length;
b) significantly homologous to a transmembrane domain of a G protein-coupled receptor; and
c) selectively inhibits the activity of a G protein-coupled receptor.

3. A peptidomimetic compound derived from a compound as in claim 1, wherein the peptidomimetic compound is generated by following the steps:
a) simulating the most probable conformations of the peptide;
b) selecting the most probable conformation of the peptide thus simulated;
c) designing and synthesizing a chemically modified analog of the selected peptide;
d) evaluating the bioactivity of the synthesized chemically modified analog of the selected peptide; and thereafter, optionally
e) designing a suitable peptidiomimetic based on the conformation of the synthesized chemically modified analog of the selected peptide.

4. A composition comprising one or more compounds of claim 1, 2 or 3 in a pharmaceutically acceptable carrier.

5. A method for inhibiting G protein-coupled receptor mediated processes comprising administering a pharmaceutically effective amount of a composition according to claim 4 effective to inhibit said G protein-coupled receptor from binding its agonist, and a pharmaceutically acceptable carrier.

6. A method of identifying novel peptide antagonists of G protein-coupled receptors comprising the steps:
a) identify and define the peptide-lead based on a transmembrane domain of the G protein-coupled receptor of interest; and
b) test the ability of the peptide-lead to inhibit the activity of the G protein-coupled receptor from which it was derived.

7. A DNA sequence coding for a compound as in claim 1.

8. An expression vector comprising the DNA sequence of claim 7.

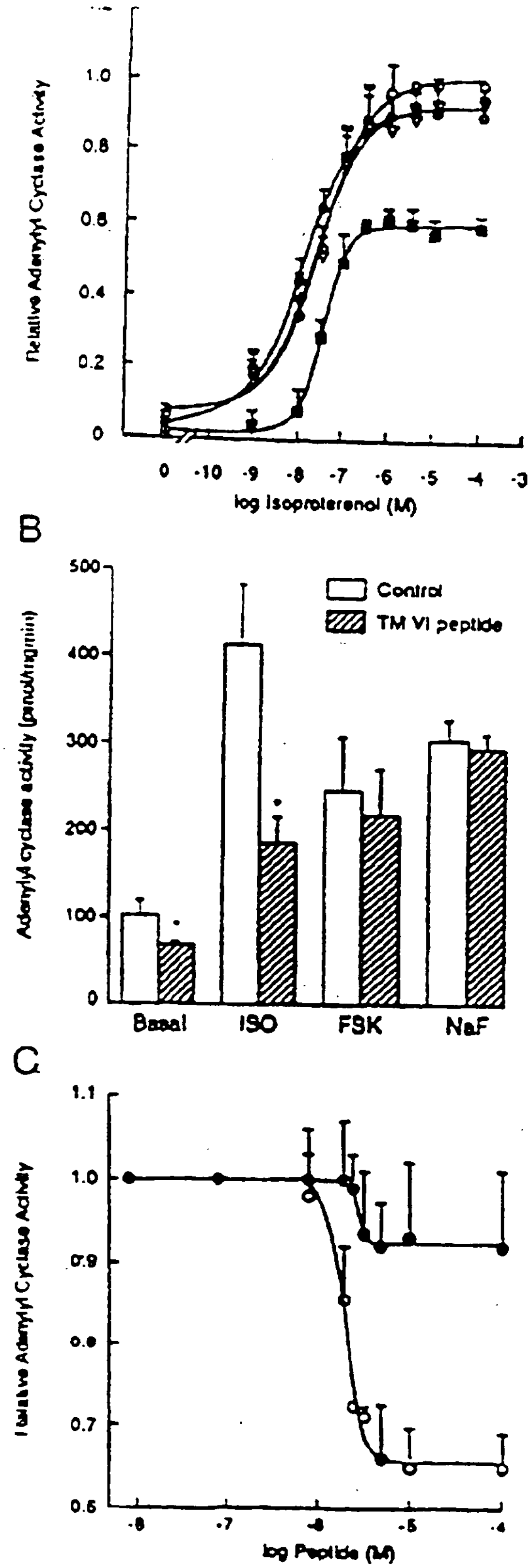
9. A method of making a peptide that is significantly homologous to a transmembrane domain of a G protein-coupled receptor that selectively inhibits the activity of a G protein-coupled receptor comprising the steps producing a biologically active peptide demonstrating G protein-coupled antagonist activity comprising:
a) constructing an expression vector containing a DNA sequence encoding the biologically active peptide;
b) transforming a bacterial host cell with the vector;
c) and culturing the transformed host cell such that the peptide is expressed; and
d) recovering the biologically active peptide.

10. A method of treating a living being with a selective G protein-coupled receptor antagonist comprising the steps:
a) preparing a compound comprising a peptide, fragment, analog or derivative of a transmembrane domain sequence of a protein-coupled receptor, said compound having the ability to selectively inhibit the function of the G protein-coupled receptor;
b) combining the synthetic peptide with a delivery vehicle; and
c) administering to a living being a G protein-coupled receptor inhibiting amount of the agent.

11. A method for preventing or treating neurological disorders involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 4 effective to prevent or treat said disorder.

12. A method for preventing or treating genetic disorders involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 4 effective to prevent or treat said disorder.

13. A method for preventing or treating disease involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 4 effective to prevent or treat said disease.



Relative Adenylyl Cyclase Activity
1.0
0.8
0.6
0.4
0.2
0
0
-10
-9
-8
-7
-6
-5
-4
-3
log Isoproterenol (M)
B
500
400
300
200
100
0
Adenylyl cyclase activity (pmol/mg/min)
Control
TM VI peptide
Basal
ISO
FSK
NaF
C
1.1
1.0
0.9
0.8
0.7
0.6
Relative Adenylyl Cyclase Activity
-8
-7
-6
-5
-4
log Peptide (M)

FIGURE 1

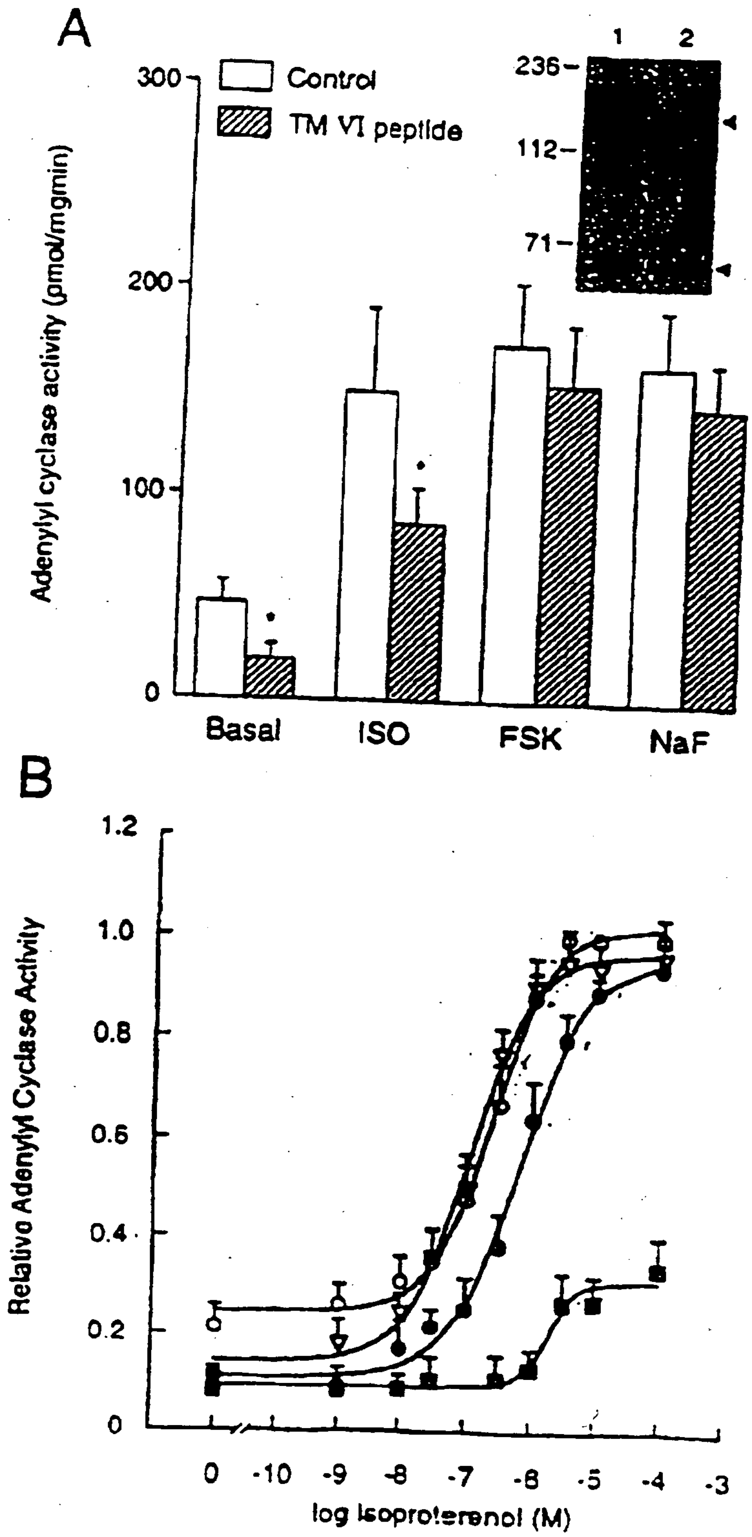

A
Control
TM VI peptide
1
2
236
112
71
300
200
100
0
Adenylyl cyclase activity (pmol/mgmin)
Basal
ISO
FSK
NaF
B
1.2
1.0
0.8
0.6
0.4
0.2
0
Relative Adenylyl Cyclase Activity
0
-10
-9
-8
-7
-6
-5
-4
-3
log Isoproterenol (M)

FIGURE 2

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: PEPTIDE ANTAGONISTS DERIVED FROM THE TRANSMEMBRANE DOMAINS OF G PROTEIN-COUPLED RECEPTORS

(57) Abstract

This invention relates to peptides and peptidomimetic compounds that act as antagonists against G protein-coupled receptors (GPCRs). Novel short peptides, derived from the transmembrane domains of GPCRs, ranging in size from about 15-20 amino acid residues, can be used as model peptides (peptide-leads) to design novel peptides and peptidiomimetic compounds that antagonize the activity of the same or closely related GPCRs from which they are derived. A lead peptide which may also be a preferred peptide antagonist for the human $\beta 2$-adrenergic receptor is NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 97/00263

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 A61K38/17 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER T ET AL: "Differential regulation of G protein alpha-subunit GTPase activity by peptides derived from the third cytoplasmic loop of the alpha-2-adrenergic receptor." FEBS LETTERS 365 (1). 1995. 13-17. ISSN: 0014-5793, XP002045148 see page 14, paragraph 3.3 - page 15; table 1 ---	1-13
Y	BIHOREAU C ET AL: "MUTATION OF ASP-74 OF THE RAT ANGIOTENSIN II RECEPTOR CONFERS CHANGES IN ANTAGONIST AFFINITIES AND ABOLISHES G-PROTEIN COUPLING." PROC NATL ACAD SCI U S A 90 (11). 1993. 5133-5137. CODEN: PNASA6 ISSN: 0027-8424, XP002045149 see the whole document ---	1-13

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1997	11. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CASCIERI M A ET AL: "Molecular characterization of a common binding site for small molecules within the transmembrane domain of G - protein coupled receptors." JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS, vol. 33, no. 4, August 1995, pages 179-185, XP002045150 see the whole document ---	1-13
A	UNDERWOOD, DENNIS J ET AL: "Structural model of antagonist and agonist binding to the angiotensin II, AT1 subtype, G protein coupled receptor" CHEM. BIOL. (1994), 1(4), 211-21 CODEN: CBOLE2;ISSN: 1074-5521, XP002045151 see the whole document ---	
A	GUAN X-M ET AL: "Determination of Structural Domains for G Protein Coupling and Ligand Binding in beta-3- Adrenergic Receptor." MOLECULAR PHARMACOLOGY 48 (3). 1995. 492-498. ISSN: 0026-895X, XP002045152 see the whole document ---	
A	EASON M G ET AL: "The palmitoylated cysteine of the cytoplasmic tail of alpha-2A- adrenergic receptors confers subtype-specific agonist-promoted downregulation." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 91 (23). 1994. 11178-11182. ISSN: 0027-8424, XP002045153 see the whole document ---	
A	PEREZ D M ET AL: "Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein - coupled receptor." MOLECULAR PHARMACOLOGY, vol. 49, no. 1, January 1996, pages 112-122, XP002045154 see the whole document ---	
P,X	HEBERT T E ET AL: "A peptide derived from a beta-2- adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation." JOURNAL OF BIOLOGICAL CHEMISTRY 271 (27). 1996. 16384-16392. ISSN: 0021-9258, XP002045155 see the whole document -----	1-13

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International application No
PCT/CA 97/00263

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 5 and 10 to 13 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.